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(54) Title: REPOPULATION OF TESTICULAR SEMINIFEROUS TUBULES WITH FOREIGN CELLS

(57) Abstract

An animal harboring a non-native germ cell, its corresponding line, and the corresponding germ cells, are obtained by colonizing the testis (or testes) of a host animal with primitive cells followed by raising and/or breeding the host.

REPOPULATION OF TESTICULAR SEMINIFEROUS TUBULES WITH FOREIGN CELLS

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Field Of The Invention:

The present invention relates to animals harboring a non-native germ cell, to corresponding animal lines and germ cells, and to methods for obtaining the same.

Discussion Of The Background:

There have been many attempts to influence differentiation of developing cells by modifying the genotype of an embryo and then observing its effect on the phenotypic development pattern in the progeny. These techniques included nuclear transfer (McGrath & Solter, Science (1983) 220:1300-1302) and cell-egg fusions (Graham, "Heterospecific Genome Interaction," 1969 Wistar Institute Press, pp. 19-35, ed. Defendi). Of the two, the former has had limited success.

Another approach might be to add a stem cell(s) to an early embryo and determine its effect on development. For example, one might imagine that a stem cell from bone marrow would contribute to the population of, and thus modify the differentiation of the evolving bone marrow cells in the host embryo. To pilot these experiments, older embryo cells were introduced into young embryos resulting in modest success with colonization (Moustafa & Brinster, J. Exp. Zool. (1972) 181:193).

the modified cells introduced into a blastocyst where they participate in development and can become sperm. This technique allows very specific modification of the mouse genome and other species.

Techniques for obtaining non-human transgenic animals through the injection of DNA into eggs are also known. See e.g., Gordon et al, Proc. Nat. Acad. Sci. (USA), (1980) 77:7380-7384. These techniques however, as well as the use of ES cells noted above, are very labor intensive.

Summary Of The Invention:

Accordingly, it is an object of this invention to provide a facile method for obtaining animals harboring a biologically functional non-native germ cell, and for obtaining corresponding resultant germ cells.

It is another object of this invention to provide a facile method for obtaining new animal lines, and for obtaining corresponding resultant germ cells.

It is another object of this invention to provide animals harboring a biologically functional non-native germ cell, their progeny, and corresponding resultant animal lines and germ cells.

It has been discovered by the inventors that the above objects and other objects which become apparent from the description of the invention given hereinbelow are satisfied by the following method. The testis (or testes) of a male animal host is (are) repopulated with at least one primitive cell (e.g. a totipotent stem cell) which is not native to the host. The animal may then be bred to obtain a novel animal

Cells which can develop into more differentiated cells of two or more types are used as the primitive cells. Thus one of two types of cells can be used: (1) totipotent cells, which are cells having the potential to differentiate into any cell type, including germ cells; and (2) pluripotent cells, which are cells capable of differentiating into two or more types of cells, e.g., bone marrow stem cells, liver stem cells, kidney stem cells, etc....

An illustrative procedure for repopulating the testis (or testes) of a host is as follows. A male host animal is prepared by destroying the native germ cell population in the seminiferous tubules by a method that leaves intact (i.e., biologically functional) the supporting cells, including the Sertoli cells. Suitable known methods include physical means (e.g. radiation[1], heat[2], etc.), chemical means (e.g. cadmium[3], Busulfan®[4], etc.), but any other known techniques to selectively destroy native sperm cells can be used. Preferably radiation is used, with Busulfan@ being most preferred.

A typical Busulfan® treatment is illustrated as follows. Forty milligrams of Busulfan® are dissolved in 10 ml of dimethylsulfoxide, to which 10 ml of water are then added. An aliquot of this solution is injected intra-peritoneally into a

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[1]Withers et al. Radiation Res. (1974) 57:88-103.

[2]Gasinka et al, Neoplasma (1990) 37(3):357-366.

[3]Parizek, J. Reprod. Fert. (1960) 1:294-309..

[4]1,4-Butanediol dimethylsulfonate esters; e.g., 1,4-bis(methanesulfonoxy)butane; 1,4-di(methanesulfonyloxy)butane; 1,4-di(methylsulfonoxy)butane; methanesulfonic acid tetramethylene ester; tetramethylene bis(methane sulfonate). See Bucci et al, Mutation Res. (1987) 176:259-268.

the host, spermatogonia may be first transferred to the thymus of the host to induce tolerance. See, Posselt et al, Science (1990) 249:1293-1295.

Alternatively, cells from an animal of the same strain as the recipient host can be used, or an immunodeficient host can be used. For an example of the latter, inbred strains of mice (e.g. SCID mice or nude mice) bred to act as recipients of sperm cells can be used. As an example of the former 129/SV mice segregating the c and c^h allele for albinism can be maintained. Homozygotes and compound heterozygotes for these alleles are easily distinguished by coat color. A Busulfan®-treated homozygote for the one allele can receive donor cells from a homozygote for the other allele. If the recipient animal thereafter gives rise to offspring showing the donor-type coat color marker, this is evidence that the donor cells gave rise to functional sperm.

The primitive cells used in accordance with the invention can come from other individuals (including both the same and other species) or *in vitro* culture. Examples of primitive cells that can be used include totipotent stem cells, embryonal carcinoma cells, embryonic stem cells, sperm cells from other males (e.g. juvenile males with high levels of primitive sperm cell types), primordial germ cells, other primitive cells, etc.. Primitive sperm cells from seminiferous tubules, embryonic stem cells grown in culture, or primitive cells from body organs are prime candidates. The use of female (XX) cells is also within the scope of the present invention.

These primitive cells may be obtained in accordance with known procedures. For example, spermatogonia stem cells may

native cells possessing artificially induced mutations or variations.

The selected primitive cells are then introduced into the individual tubules. For example, a prepared male can be anesthetized and the testis (or testes) surgically exposed. By micromanipulation methods a thin glass needle is introduced into exposed tubules, one after another, and each tubule is injected with a solution containing the primitive cells being used to colonize the tubule. Alternatively, the primitive cells can also be introduced by injecting into other parts of the tubular system e.g. the lumen of the rete testes. To inject the rete testes one may use either fine stainless-steel needles or fine pulled-glass capillaries loaded with donor primitive cells. A micromanipulator is used to direct the tip of such an instrument to penetrate the rete testis. The cells are expelled and will back-fill the seminiferous tubules.

Other systems may also be suitably used for introduction of the cells. These include surgical techniques to sever the seminiferous tubules inside the testicular covering, with minimum trauma, which allow injected cells to enter the cut ends of the tubules. For example, a fine surgical thread is circled about a number of tubules, and then drawn tight, severing the tubules. A donor-cell suspension is then injected into the testis.

Alternatively neonatal testis (or testes), which are still undergoing development, can be used. Here, a surgical procedure to expose the neonatal testis (or testes) for injection of new cells is used. Neonatal mice are chilled on ice for anesthetization. The tiny testes are surgically exposed, and a small bladed-instrument is used to disrupt the

30 to 60 days, and another 10 to 15 days is needed for epididymal maturation of spermatozoa. See, e.g., "Reproduction in Domestic Animals", 4th ed., Acad. Press (1991), ed. P. Cupps. Therefore, depending on the species used, males that have received primitive cells in accordance with the invention can be examined beginning about two months after cell transfer.

The present invention is applicable to any species of animals, including human, in which the male has testes, including but not limited to transgenics. The invention is also not limited to mammalian species. It can be used to provide animals and animal lines of many types with a single, or many, novel genetic modification(s) or novel characteristic(s). The animals to which the present invention can be applied include animals, such as rodents (e.g., mice, rats, etc...), which can be modified to permit their use in cellular diagnosis or assays. The present invention may also be advantageously applied to farm animals such as domesticated ruminants or fowl (e.g., cattle, chickens, turkeys, horses, swine, etc...), to imbue these animals with advantageous genetic modification(s) or characteristic(s).

Once an initial fertilization event is achieved and the resulting offspring is fertile, the animal line with its novel genetic modification or characteristic is established, with the novel genetic modification or characteristic being present in both male and female offspring. Thus, in accordance with the invention one may produce an animal harboring, in its testes only, a biologically functional germ cell which is not native to that animal by repopulating its testicular seminiferous tubules. This (parent) animal can produce

In accordance with the present invention, spermatogonia stem cells can be genetically modified and then transferred to recipient testes. The valuable genetic traits present in the resultant germ cells can be passed onto the (transgenic) progeny of the recipient stud. This particular application of the present invention is particularly important to the genetic engineering of large agricultural animals.

The present invention also has applications in gene therapy, including human gene therapy. For example, a patient with a deleterious genetic trait could undergo a testicular biopsy. Isolated stem cells can be genetically modified to correct the deleterious trait. The patient then undergoes a treatment to remove the remaining germ cells from his testes, for example by specific irradiation of the testes. His testes (now devoid of germ cells) can then be recolonized by his own, genetically-corrected, stem cells. The patient can then father progeny free from the worry that he would pass on a genetic disease to his progeny.

Conventional cell markers, such as surface antigens or internal enzymes, can be employed in the transferred primitive cells to facilitate detecting their presence in biopsy specimens of the testes. The presence in the ejaculate of spermatozoa with the characteristics of the marker is a reliable indication of success. Thus, in accordance with a preferred embodiment, at least one genetic marker is preferably used to distinguish the introduced cells from residual sperm that might arise from the host male. For example, transgenic mice strains that can produce a characteristic stain in sperm cells which serve as donor marker cells can be used. The promoter from a gene active in developing sperm cells (Zfy-1 or a homolog) is used to drive

development to later stages of spermatogenesis of cells transferred in accordance with the invention and show the cross-strain utility of the invention, and thereby provides strong evidence of the cross-species potential of the present invention.

Introduction:

Foreign potential stem cells for transfer were isolated from the testes of very young male mice, usually between 3 to 10 days of age. These cells carried a reporter or marker gene encoding the *E. coli* ß-galactosidase (lacZ) gene. This gene is not normally present in the mouse genome. However, if a cell contains the gene, it will make the enzyme ß-galactosidase. In the presence of the reagent X-gal, the cell will then stain blue. In addition, the transgene in these cells will only be active in late stages of spermatogenesis, in the round spermatid and later stages, because the lacZ structural gene is under control of a promoter or activating DNA sequence designated ZF. These late stages of spermatogenesis are not present in the neonatal testes. Therefore the transgene is not active in neonatal testes and these cells cannot be stained blue nor do any of the cells have the appearance of mature spermatozoa at this early age. As a result of this experimental procedure, the transferred cells must not only survive, but they must undergo differentiation and development to become late stages of spermatogenesis in order to stain blue. Furthermore, the transferred cell descendants can easily be distinguished from any endogenous sperm cells of the recipient host mouse (should any be present), because these endogenous cells will not stain blue.

differentiation of transferred cells. Furthermore, these mice are immunologically incompatible with the donor cell strain of mice because the W-mice are of C57 background and would not be tolerant of the SJL antigens on the surface of cells containing the ZF-lacZ transgene.

3. Inbred mice of the 129/SV strain. These mice are immunologically incompatible with the donor cells because the cells contain C57BL/6 and SJL antigens and 129/SV mice would recognize both strains as foreign. Foreign cells would be rejected and destroyed by a mouse if present in a normal environment, such as a skin or organ graft. However, some parts of the testis, particularly the inner region of the seminiferous tubules, is considered to have a degree of immunological privilege (i.e. They do not reject foreign tissue as readily as other body locations. The uterus is the best example of an immunologically privileged location, since it does not reject the fetus carrying the male antigens).

The implementation and use of the invention has substantiated in the following examples, and the results obtained provided proof of several important aspects of the invention. The examples describing implementation of the invention are as follows.

Experimental:

1. Primitive stem cells or spermatogonia can be transferred from one animal into another host animal and the donor cells will survive, divide and differentiate. Cells were taken from neonatal testes of hybrid mice (C57BL/6 x SJL) containing the ZF-lacZ transgene, and these cells were microinjected into the seminiferous tubules of Busulfan®

Furthermore, this mutant animal is incapable of generating sperm cells. In this embodiment of the invention the donor cells have colonized a completely sterile testis in an animal that is not immunologically tolerant of the donor mouse strain. The testis environment has provided an immunological protection as anticipated in the invention application. The examination of this testis was performed 120 days after donor cell transfer indicating that cell survival, division and differentiation continues in the host for a long period, probably until death.

3. In the third implementation of the invention, the same type donor cell was transferred into the seminiferous tubule of a 129/SV inbred mouse. The mouse had previously been treated with Busulfan® to destroy endogenous sperm cells. The result is shown in Figure 6. Again the donor cells (stained blue or dark) have colonized this tubule. The differentiation has not proceeded as far as in the examples above; perhaps, because the transfers were among the first performed while improvements were still underway. However, this example demonstrates tolerance of foreign cells in the tubule despite very strong immunological differences. The examination of this testis was performed 110 days after donor cell transfer, indicating a very long period of donor cell survival.

* * * * *

Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

9. The method of Claim 8, wherein said step (i) comprises subjecting said tubules to a physical treatment.

10. The method of Claim 9, wherein said physical treatment is radiation treatment.

11. The method of Claim 8, wherein said step (i) comprises subjecting said tubules to a chemical treatment.

12. The method of Claim 11, wherein said chemical treatment is treatment with Busulfan®.

13. The method of Claim 8, wherein said supporting cells comprise Sertoli cells.

14. The method of Claim 7, wherein said primitive cells are totipotent stem cells.

15. The method of Claim 8, wherein said step (ii) comprises injecting a solution containing said primitive cells into said tubules or another part of the tubular system of said testes.

16. The method of Claim 15, wherein said animal is a farm animal.

17. A method for obtaining a germ cell comprising raising or breeding an animal of Claim 1 and collecting at least some of its germ cells.

18. A germ cell obtained from the animal of Claim 1.

24. The animal of Claim 2 wherein said mammal and said germ cell are of different mammalian species.

25. The animal of Claim 4 wherein said germ cells originate from a stud animal.

26. A method for the gene therapy of an animal in need thereof, comprising the steps of:

(i) destroying the endogenous germ cell population in the seminiferous tubules of said animal, leaving supporting cells in said tubules biologically functional; and

(ii) colonizing said tubules with stem cells free of the deleterious genetic trait(s) creating said need.

27. The method of Claim 26 wherein said animal is a human.

28. The method of Claim 26 wherein said animal is an agricultural animal.

29. A non-human animal harboring in its seminiferous tubules a biologically functional non-native germ cell, obtained by a process comprising the steps of:

(i) destroying the endogenous germ cell population in the seminiferous tubules of said animal, leaving supporting cells in said tubules biologically functional; and

(ii) colonizing said tubules with primitive cells which are non-native to said animal.

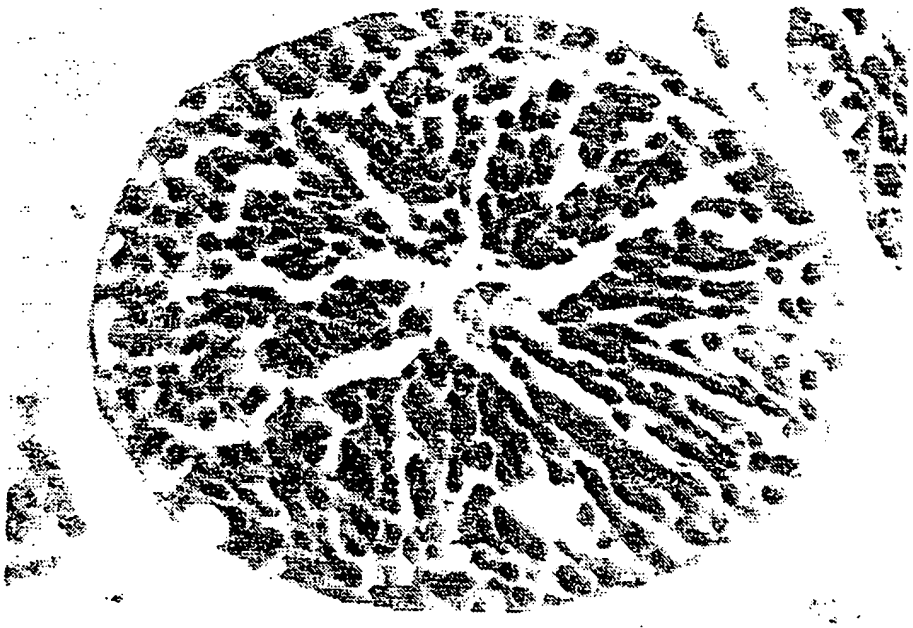

FIG. 1

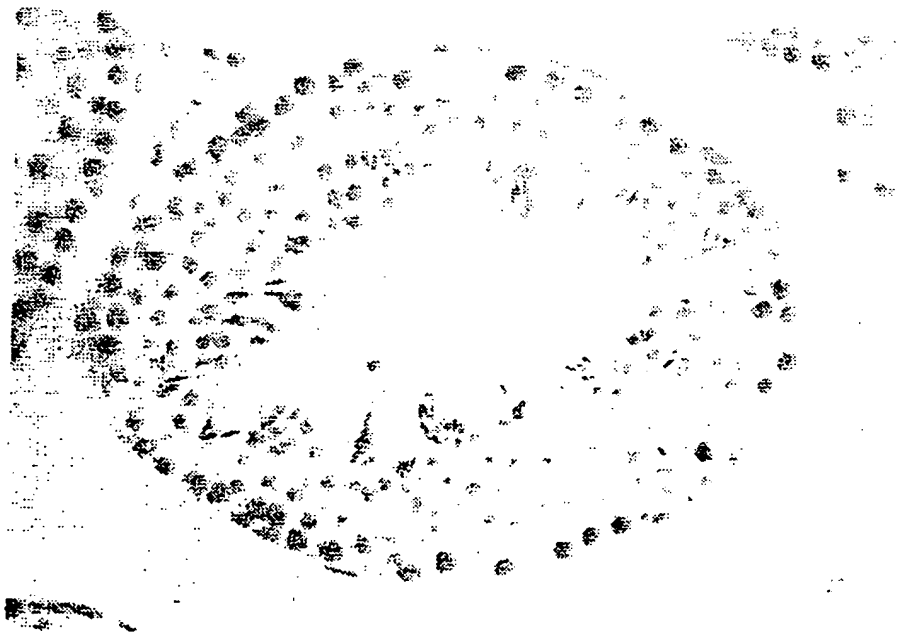

FIG. 2

FIG. 5

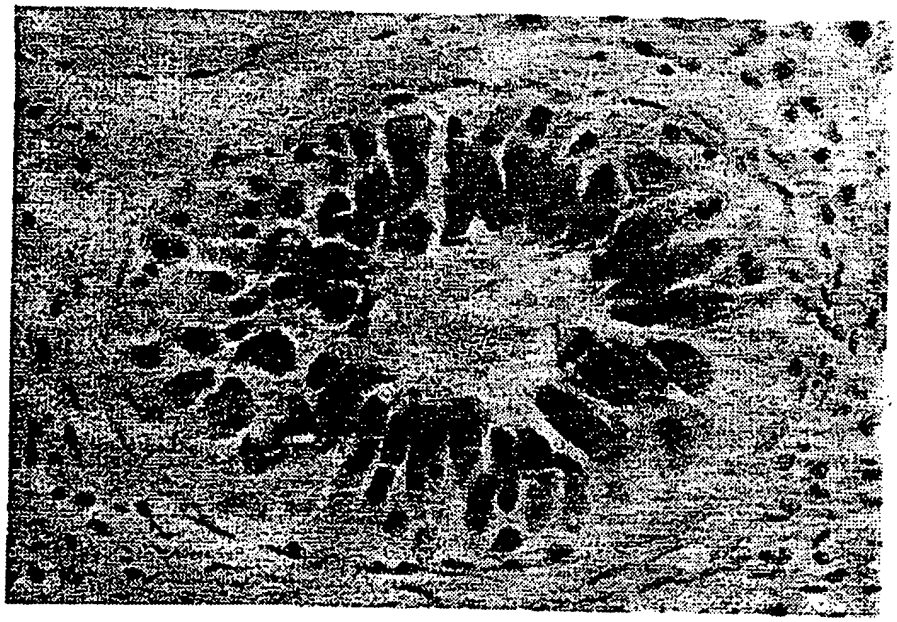

FIG. 6